Identification of genes involved in the regulation of sensory organ precursor formation in *Drosophila melanogaster*

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Molecular Genetics in the undergraduate college of The Ohio State University
by

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Abstract

Defects in early neurogenic development are associated with a wide range of pathological conditions. The Fred protein, a transmembrane IgC2 protein, is necessary for normal development and critical for the Notch signaling pathway. Using *Drosophila melanogaster* as the model, the regulatory roles of other genes can be studied that exhibit functional interactions with these genes. Macro- and micro-chaeta sensory bristles (large and small bristles) cover the adult fly and provide an avenue to study cell fate. As each bristle originates from an individual sensory organ precursor cell, the presence or absence of sensory bristles indicates sensory organ precursor cell differentiation. From an RNAi mini screen of an array of candidate genes, 2 genes, *cullin-2 (cul-2)* and *tumbleweed (tum)*, were identified and exhibited a phenotype similar to that of *fred*, specifically a loss of function. cul-2 is a scaffold protein for ubiquitin ligase, while tum is involved in GTPase inactivation. An additional aim of this study is to understand how these two genes interact within the *fred* pathway. The two genes were identified using the inducible RNAi / GAL4/ UAS system. The GAL4/UAS system allows one to induce gene specific RNAi in specific tissues and at defined developmental times. Observing the phenotypical consequences of this down regulation occurs at two levels: (1) the adult animal, and (2) in imaginal wing disc of late 3rd instar larvae. Additional studies have been started to further define the phenotype of these genes and their respective interactions with *fred* gene function. Initial experimental observations suggest that *tum*, indeed, may have additional roles in the regulation of muscle attachment sites. Furthermore, double mutations of *cul-2* and *tum*, *tum* and *fred*, *tum* and *sc* are being analyzed to determine potential epistatic relationships.

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Introduction

Background

The adult fly is covered in macrochaeta and microchaeta (large and small bristles), which are sensory organs that help the animal sense its environment. Sensory organs arise from (sensory organ precursors) SOP cells in the third instar larve. Three groups of genes regulate sensory organ precursor formation according to the prepattern hypothesis: prepattern, proneural and neurogenic genes. Prepattern genes mark overlapping regions of gene expression (Culi et al., 1998). Proneural genes are expressed in so-called proneural clusters and result from prepattern gene function (Campos-Ortega, 1993). Subsequently, proneural genes interact with Notch signaling to limit neurogenic potential to a single SOP in each proneural cluster (Figure 1).

Previous work in the lab has identified a gene *friend of echinoid* (*fred*) (Chandra et al., 2003), which is similar in sequence to *echinoid* (Ahmed et al., 2003). *Echinoid* is an immunoglobulin C2-type cell-adhesion molecule (IgC2), which is involved with the Delta ligand in the Notch pathway (Rawlings et al., 2003). Preliminary investigation in the Vaessin lab suggests that there is an earlier regulating step prior to the prepattern genes. This earlier process involves a requirement of *fred* function (Chandra). Subsequent work in the lab was enacted towards the identification of fred interaction or downstream genes. The purpose of my study is to better understand which genes, from a collection of genes that were found to be miss-expressed early on in neurogenesis in a *fred* microarray study, function.

What is *cullin-2*?

cul-2 is located on the second chromosome and is a member of the cullin gene family that plays a critical role in the ubiquitination pathway. Cullin 1-7 are scaffolding proteins that make up the cullin-RING E3 ubiquitin ligase (CRL). The ubiquitin-proteasome system works by

attaching ubiquitin to an E1 activation enzyme in an ATP dependent reaction. This ubiquitin subsequently is transferred to an E2 conjugation enzyme. The E3 ligase binds both substrate and E2 which results in mono or polyubiquitination that will signal whether to be degraded by the 26S proteosome (Sarikas, 2011). Ubiquitination of proteins is essential for many protein signaling processes some of which include tumor suppression, cell growth and signal transduction. There are six *cullin* genes in the Drosophila genome (Hudson et al., 2010)**,** and eight members in mammals. *cul-2* function is currently not well understood in mouse or *Drosophila melanogaster*. In *C. elegans cul-2* is involved in a range of physiological functions.

What is *tummbleweed*?

tum also known as RacGAP50C is located on the second chromosome. *tum* mutations disrupt GTPase Activating Protein (GAP) (Goldstein et al., 2005). GAP and guanine nucleotide exchange factors (GEF) work as genetic switches in the cell to relay signals by either activating or deactivating GTPase (Somers et al., 2003). The *ras* gene, which is a monomer GTPase, is mutated in 1 in 5 of all human cancers (Alberts et al., 2008). It is proposed that *tum* serves as a scaffolding protein (Goldstein). Another known function of RacGAP50C is that it regulates cytoskeleton required for cytokininesis, and is required to limit axon growth (Goldstein 2005). It connects the contractile ring to cortical microtubules at the site of furrowing in dividing cells and negatively regulates the wingless pathway during Drosophila embryonic development, and is required for neuroblast proliferation and limits axon growth (Goldstein).

DNA microarray

DNA microarray technology enables the study of genome wide patterns of gene expression. The Vaessin lab had previously completed a microarray for *fred-RNAi* mRNA and found 62 downregulated genes and 72 upregulated genes (unpublished results). Furthermore, an

RNAi mini screen was performed on microarray confident genes and from a fred yeast two hybrid screen. RNAi lines, where available, have been obtained for these genes and used for phenotype observations.

Fifteen genes were initially studied by creating fly crosses and observing phenotypes at the adult and wing disc level. *cul-2* and *tum* were two genes identified to display adult phenotypes in line with possible role in early neurogenesis, and also displaying ectopic (outside of normal expression) SOP formation in imaginal wing discs. In this thesis I present the identification of *cul-2* and *tum* and evidence that *cul-2* and *tum* are two new novel genes in early neurogenesis.

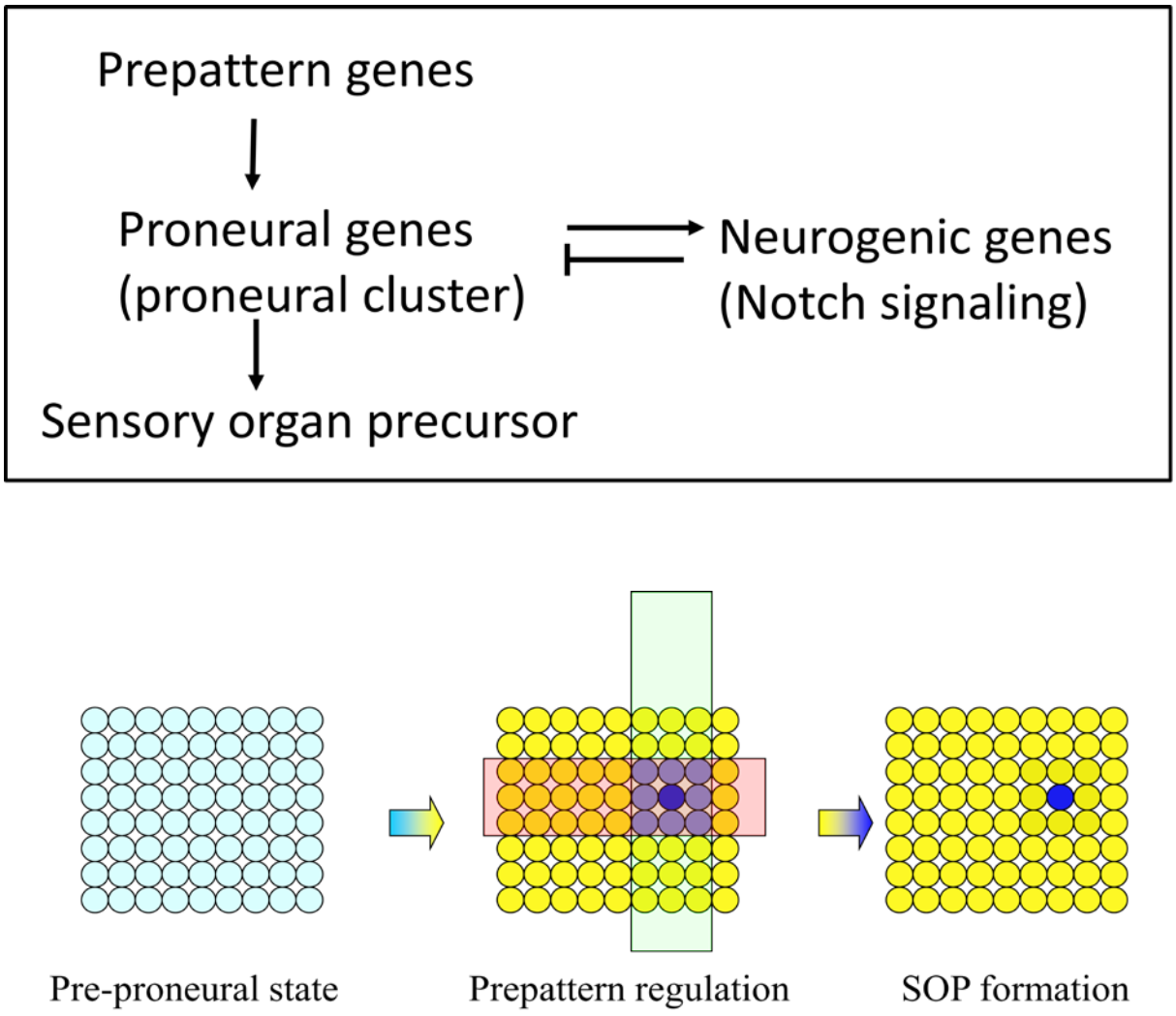

Figure 1: Prepattern hypothesis

Approach/ Material and Methods

Stocks used

Fifteen UAS-CG-RNAi lines from Dr. Maki Asano and Vienna fly center were used for initial identification crosses (Figure 2). Other stocks used: *pnr-GAL4, ap-GAL4, c765-GAL4, en-GAL4, neurA101-lacZ,* hsFLP; FRT42tum^{347}, FRT42GFP, *tubP-GAL80ts. UAS-fred RNAi, sca-lacZ.*

Fly chromosomes and balancers

Flies have 4 pairs of chromosomes an X/Y pair and three autosomal chromosomes. Since a fly is diploid and inherits one set of chromosomes from each parent, a tracking system is needed. Balancers are used for this purpose. To accomplish this, balancer chromosomes generally have: (1) multiple chromosome aberrations such as inversions and translocations, (2) at least one dominant marker gene, and (3) frequently one recessive lethal mutation (Lindsley and Grell, 1972). Using balancers allows one to visualize the genotype by looking at the phenotype (Ashburner, 1989). An example of a balancer is Curly derivative of Oster (CyO). If a fly inherits this marker it will have curly wing as a dominant tracking phenotype. Another balancer called Stubble (Sb) results in a fly with shorter sensory bristles. Figure 3 depicts a hypothetical fly cross and resulting progeny to demonstrate this point.

GAL4/UAS system

The GAL4/UAS system is a borrowed genetic system from yeast. GAL4 is a yeast gene that encodes for the GAL4 protein, which is a transcription factor. Upstream Activation sequence (UAS) is a GAL4 binding site on DNA only found in yeast that is the target of GAL4. Both GAL4 and UAS by themselves do not do anything, but in combination GAL4 can bind UAS and drive expression of coding sequence downstream of the UAS sequence. In flies we accomplish

this by transgenic means. One fly stock in which the GAL4 gene has been introduced is crossed with a UAS fly stock. A proportion of the offspring will have both GAL4 and UAS. Furthermore, GAL4 lines are used that express GAL4 in a specific spatial/temporal developmental context. The UAS sequence is part of a construct that also contains a gene of interest to be miss-expressed (Figure 4). If this gene of sequence is an RNAi construct, via a Palindrome hairpin sequence the target gene will be down regulated (Brand and Perrimon, 1993).

X-gal staining for wing discs

β-galactosidase activity can be detected by X-gal. This method was used on all fifteen CG lines. *en-GAL4>> UAS-CG-RNAi* and *pnr-GAL4;A101>>UAS-CG-RNAi*. The resulting flies are collected under CO_2 and stored in 96% ethanol. Pictures are compiled using a dissection microscope. Additionally, third instar larvae are collected for X-gal staining. Larvae are dissected in ice cold PBS for no more than 10 minutes. Fixation of larvae in 1% glutaraldehyde is done for 10 minutes. Samples are washed three times with PBT (PBS with 1% tween-20) 3 minutes each. The solution is removed and 10 ul of 10% X-gal solution in 490 ul X-gal staining buffer is added. X-gal staining buffer is composed of 160 mg of Potassium Ferricyanide Crystalline (5mM), 210 mg of Potassium Ferricyanide Trihydrate (5mM), 20 mg of Magnesium Chloride (2mM), 100 ml PBS, and 100 ul of Tween-20. The reaction proceeds at room temperature overnight. Reaction is stopped by washing 3 times for 3 minutes each with PBT. Finally, discs are isolated and mounted in aquamount.

Digoxygenin-labeled RNA probe

Protocol for synthesizing DIG-labeling transcription reaction. Primers for *cul-2* were combined with genomic DNA. The antisense primer was constructed with a T7 promoter. Each 10 ul reaction should contain: DNA 3.5 ul, mMATP 0.75 ul, 0.75ul mMGTP, 0.75ul mMCTP,

0.50ul mMUTP, 10x transcription buffer, 1.3 ul digoxygenin UTP, Roche MEGAscript T7 RNA polymerase. Set at 37°C for 6 hrs and then stored overnight at 4°C.

***cul-2 in-situ* hybridization**

In-situ hybridization allows one to visually locate the messenger RNA in the tissue. This three-day process was used for *cul-2*. Tissue can be dehydrated in alcohol in a series of steps (25%, 50%, 75% 96% Et-OH) after the first fixation and stored at -20°C on the first day.

Day 1: Dissect third instar larvae and invert in ice cold PBT in less than 10 minutes. Fixate the tissue with 4% formaldehyde for 20 minutes. Wash in PBT 3 min each, 4 times. Exchange solution for 500 ul of PBT with 2 ul of proteinase K. Quick wash 4 times in PBT to stop the reaction by inverting after each exchange. Post fixation with 4% formaldehyde 20 minutes followed with washing 4 times 3 minutes each in PBT. Exchange into hybridization solution for 5 minutes, twice. Exchange again and incubate for an hour at 55°C. Remove and add 1 ul of RNA probe to 50 ul of hybridization solution. Heat to 95°C for 3 minutes, cool on ice for 3 minutes and add wing disc at 55°C overnight.

Day 2: Wash for 20 minutes each in hybridization solution at 55°C, 4 times. Washed 15 minutes each at 50% and 25% hybridiazation solution at room temperature. Washed in PBT for 7 minutes each, 5 times. Meantime rehydrate embryos (25%, 50%, 75%,100% PBT), 2 minutes each step. Block 10 minutes with PBT and 1% BSA. Add antibody AntiDig (1:500). Incubate for 1 hour at room temperature. Use this pre-incubated antibody and add to the wing discs at a final (1:4000) dilution over night at 4°C.

Day 3: Wash for 15 minutes each in PBT, 4 times; followed by AP buffer wash, 2 times. Remove last of AP buffer and add AP buffer with 4.5 ul NBT and 3.5 ul BCIP and place

in viewing dish in dark. Check the color reaction frequently. Stop reaction with a drop of ethanol. Wash with PBT and mount in aquamount.

Setting up Double mutation crosses

Double mutation crosses were performed with *tum* in *cul-2*, *tum* in *sc*, and *tum* in *fred*. Below are the crosses for the resulting genetics. Step four in the series of crosses below was established to create a stock.

UAS-tum-RNAi(II) in UAS-cul-20RNAi(III) double mutation crosses .

1) ♂ *UAS-tum-RNAi/UAS-tum-RNAi x ♀ L/CyO; Sb/tb*
2) ♂ *UAS-tum-RNAi/CyO; +/sb x ♀ L/CyO; UAS-cul-2-RNAi/Tb*
3) ♂ *UAS-tum-RNAi/L;cul-2RNAi/Sb x ♀ Sb/CyO-tb*
4) ♂ *UAS-tum-RNAi; UAS-cul-2-RNAi/CyO-tb x ♀ en-GAL4; A101/CyO-tb*
5) *UAS-tumRNAi; UAS-cul-2RNAi/ en-GAL4; A101*

UAS-tum-RNAi(II) in UAS-fred-RNAi(III) double mutation crosses

1) ♂*UAS-tum-RNAi/UAS-tum-RNAi x ♀ L/CyO; Sb/tb*
2) ♂*UAS-tum-RNAi/L; +/tb x ♀ Sco/Cyo; UR3-3/Sb*
3) ♂ *UAS-tum-RNAi/Cyo; UR3-3/Tb x ♀Sb/CyO-Tb*
4) ♂ *UAS-tum-RNAi: UR3-3/ CyO-Tb x ♀ en-GAL4: A101/CyO-Tb*
5) *UAS-tum-RNAi: UR3-3/ en-GAL4:A101*

UAS-tum-RNAi(II) in UAS-sc-RNAi(III) double mutation crosses

1) *♂UAS-tum-RNAi/UAS-tum-RNAi x ♀L/CyO; Sb/Tb*
2) *♂UAS-tum-RNAi/Cyo; +/Sb x♀ L/Cyo; Sc-RNAi/Tb*
3) *♂UAS-tum-RNAi/Cyo; UAS-Sc-RNAi/ Sb x ♀ Sb/ Cy-Tb*
4) *♂UAS-tum-RNAi; UAS-Sc-RNAi/CyO-Tb x ♀en-GAL4: A101/CyO-Tb*
5) *UAS-tum-RNAi; UAS-Sc-RNAi/ en-GAL4: A101*

Stock #/CG	Name
1. 104160/1034	Bicoid
2. 105101/1512	cullin-2
3. 101538/2621	Shaggy
4. 100165/2909	unknown
5. 105562/3244	C-type lectin 27kD
6. 104523/4005	Yorkie
7. 101829*/4373	Cyo6d2
8. 100906/5210	chitinase-like
9. 100281/9390	Acetyl Coenzyme A synthase
10. 107130/10079	Epidermal growth factor receptor
11. 110392/10955	Rtf1
12. 101166/12283	kekkon-1
13. 106850/13345	Tumbleweed
14. 104496/17870	14-3-3
15. 101275/34395	Nubbin

Figure 2: *UAS*-CG-*RNAi* stocks used

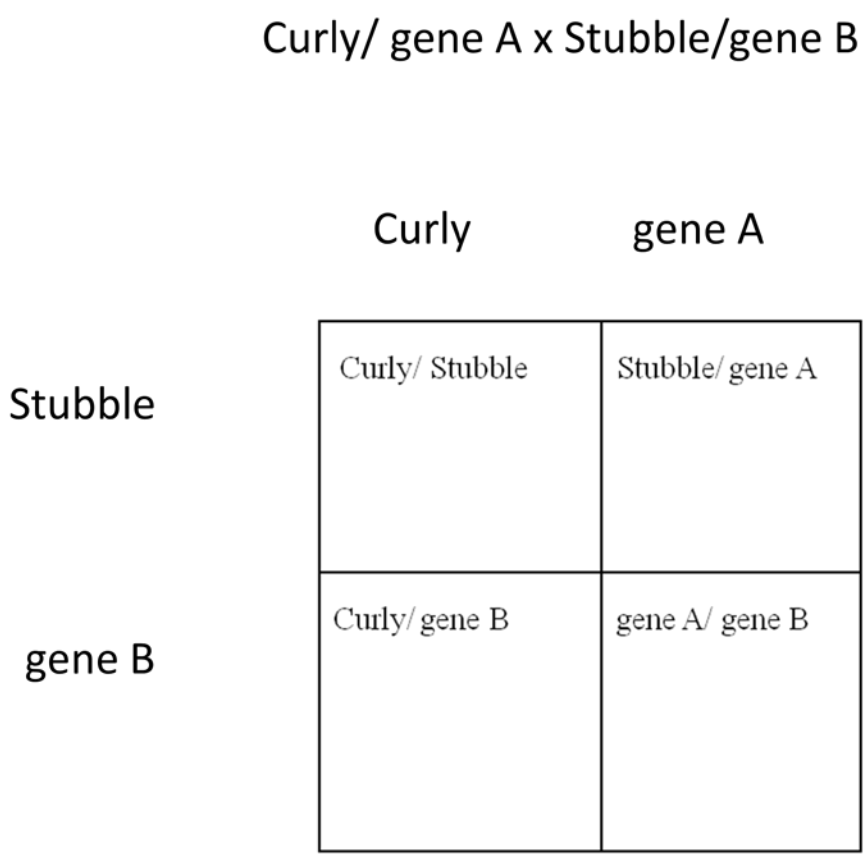

Figure 3: Fly crosses and balancers

In this hypothetical cross one fly with the dominant balancer Curly is crossed to a fly with the Stubble mutation. Four possible progeny are apparent and can be distinguished by the flies' phenotype.

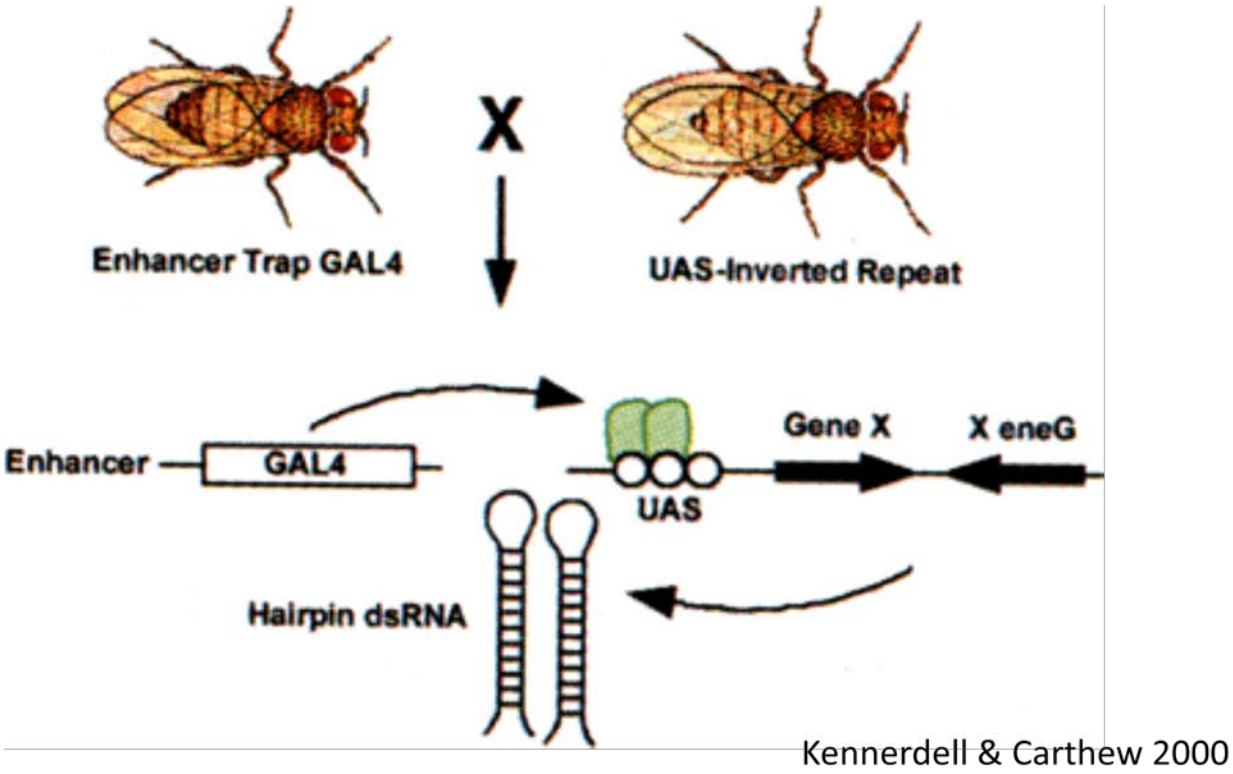

Figure 4: GAL4/UAS-RNAi system

A fly crossed to create progeny with both GAL4 and UAS will allow the UAS construct Gene X to be miss-expressed by the enhancer trap GAL4. The enhancer trap is expressed in a spatial/ temporal background. The downstream DNA coding sequence will hairpin if the order is a Palindrome.

Results

Identification of *fred* interacting genes

In order to identify genes that potentially interact with *fred,* I used the small wing phenotype of UAS-*fits*. The *fred* intercellular transmembrane domain sequence (*fits*) is construct containing parts of the *fred* protein, specifically the intercellular domains and the transmembrane domain. It was hypothesized that the genes which interact with *fred* may modify this phenotype. Two types of crosses were used to misexpress *CG-RNAi.* First, I used *ap-GAL4* misexpression to drive *UAS- fits* with *UAS-CG-RNAi* (Figure 5). *apterous* (*Ap)* is expression in a large part of the wing compartment and notum. It was observed that the *fits* animals show smaller wing phenotypes, and the *ap-GAL4 >>UAS-cul-2-RNAi* animals have smaller wings and loss of macrochaeta. The second cross *c765-GAL4* was used to miss-express *UR3-1* with *UAS-CG-RNAi* (Figure 5). *c765-GAL4 is* expressed in the wing.

Smaller wings in *c765-GAL4 >>UAS-cul-2-RNAi* were observed. These identification crosses identified another gene *tum* from the *c765-GAL4 >>UR3-1;UAS-tum-RNAi* cross (Figure 5). In this animal the wing marginal shows an increase in the number of bristles.

Cul-2 adult phenotype knockdown with drivers *pnr-GAL4, en-GAL4, ap-GAL4, and c765-GAL4*

pannier-GAL4 (pnr-GAL4), engrailed-GAL4 (*en-GAL4), apteros-GAL4 (ap-GAL4), and c765-GAL4* were used to misexpress *cul-2-RNAi* in adult animals. *pnr-GAL4* is expressed in the median notum. *en-GAL4* is expressed in the posterior compartment of the wing. *ap-GAL4* shows large expression in most of the wing and notum. *c765-GAL4* is limited to the wing. I observed missing macro and microchaetae in *pnr-GAL4;A101>>UAS-cul-2-RNAi* (Figure 6). The animals

with the *en-GAL4>>UAS-cul-2-RNAi* and *c765-GAL4>>UAS-cul-2-RNAi* showed smaller wings. *Ap-GAL4* show smaller wings and loss of bristles on the notum.

cul-2 knock-down induces ectopic SOPs outside proneural clusters

Wing disc phenotypes using A101 staining with *en-GAL4* and *pnr-GAL4* drivers. *en-GAL4* is expressed in the posterior region of the wing disc, while *pnr-GAL4* is in the distal region. *en-GAL4>>UAS-cul-2-RNAi* animals showed A101 expression indicating ectopic SOP cells. In the control no ectopic SOP were observed (Figure 7). *pnr-GAL4>>UAS-cul-2-RNAi* also showed ectopic SOP cells. These pheneotypes resember the *fred-RNAi* phenotype.

cul-2 in-situ

fred-RNAi knock down compared to *cul-2-RNAi* knock down displays stronger phenotypes in the notum. To determine the gene expression of the *cul-2* gene, *in-situ* hybridization was performed. In the first instar larvae, general uniform expression in wing discs was found. In the second day third instar larvae, the wing discs show less uniform expression with higher levels of expression in the wing margin. *cul-2* mRNA (Figure 8) shows that lower levels in some regions of the wing disc would support the observation of weaker phenotypes seen in the notum of the animals

***tum* adult phenotypes knockdown with drivers**

In order to determine the adult phenotype with RNAi, *tum-RNAi* lines were crossed with *en-GAL4* and *pnr-GAL4*. In the progeny of these crosses, smaller thorax, smaller wings and loss of macro and microchaetae existed (Figure 9).

tum* wing disc phenotype resembles *cul-2* more than *fred

Using A101 neurogenic marker in the wing disc for SOP cells the disc phenotype was shown for tum (Figure 10). The genetics were *en-GAl4>>UAS- tum-RNAi* and *pnr-GAl4>>*

UAS-tum-RNAi show phenotypes that closely resemble the *cul-2-RNAi* wing disc phenotypes. Even more so than the *fred-RNAi* wing disc phenotype.

tum GAL80 temperature shift (ts) study

tum is shown to have an early function in development. Any potentially later function of tumbleweed would be masked by earlier events when using the GAL4/UAS-RNAi technique, and has not yet been studied. GAL80ts (mutation of normal GAL80) is not functional as 29°C degrees and is unstable (McGuire et, al., 2003). Here the GAL80ts/GAL4/UAS technique was used to study *tum* interaction at different stages of development. The mechanism works as such: GAL80ts encodes a protein in the nucleus that binds to the GAL4 protein. This inhibits the transcription machinery to be recruited. At the restrictive temperature GAL80ts loses its intrinsic function and no longer works (McGuire). In the experiment flies were created with the genotypes of either *GAL80ts-/pnr-GAL4>>UAS-tum-RNAi* (Figure 11) or *GAL80ts -/c765-GAL4>>-UAS-tum-RNAi* (Figure 12) and raised at 19°C through early stages in development. The flies were then transferred to 29°C temperature setting where GAL80ts no longer inhibits GAL4, and thus, GAL4 drives *UAS-tum-RNAi* expression at a later time in development. This enables one to test for functions of *tum* at later stages of development.

"Regular suppression" with GAL80ts driven by *c765GAL4* shows bigger wings with thicker bristles in the wing marginal and different positioning of the wings compared to "unmodified suppression" with no GAL80ts. "Regular suppression" with GAL80ts driven by *pnr-GAL4* on the other hand showed bulges in the epidermis. See "*tum* has a later function in development" for discussion.

tum mosaic study

FRT/FLP system: heat shock is used to induce flip protein, not by UAS, but with the Hsp70 promoter. Heat shock induces Flip protein expression (Theodosiou and Xu, 1998). A mosaic is a mixture of cells that have different genotypes within an organism. In this study transgenic flies with the yeast recombinase *flip* gene is combined with its target FRT to create mosaic flies. Homologous chromosomes undergo mitotic recombination when the animals are heat shocked and FLIP is expressed. The animals were heat shocked in a water bath each day for 45 minutes at 38°C to induce recombination. Due to time limitations studies could not be performed in the wing disc because all crosses must be created to have each animal with the correct, corresponding, markers. The following genetics was used to create *tum* clone: hsFLP; FRT42tum^{347}/cy-tb x FRT;GFP/Cy. In this pilot experiment, I observed animals that had multiple bristles from one socket, in addition to marginal wing bristles and mild rough eye phenotype (Figure 13).

Double mutations

Formation of SOPs requires a function of the proneural *scute (sc)* gene. In order to determine if s*cute* can suppress or reduce the *tum* phenotype, I created a series of crosses with *UAS-tum-RNAi* and *UAS-cul-2-RNAi* taking advantage of fly balancers, figure not shown. My hypothesis is that *sc* can reduce ectopic SOP in the wing disc because *sc* is necessary for SOP development. Furthermore, *en-GAL4* was used to for misexpression. The second double mutation *tum* in *cul-2* was spawned to gain insight into any possible interaction between *cul-2* and *tum*. The last combination of double mutations created was *tum* and *fred*. The purpose of these crosses was to determine the potential epistatic relationship of these genes. I observed ectopic SOP in the wing discs and smaller wing. See discussion for an depth analysis.

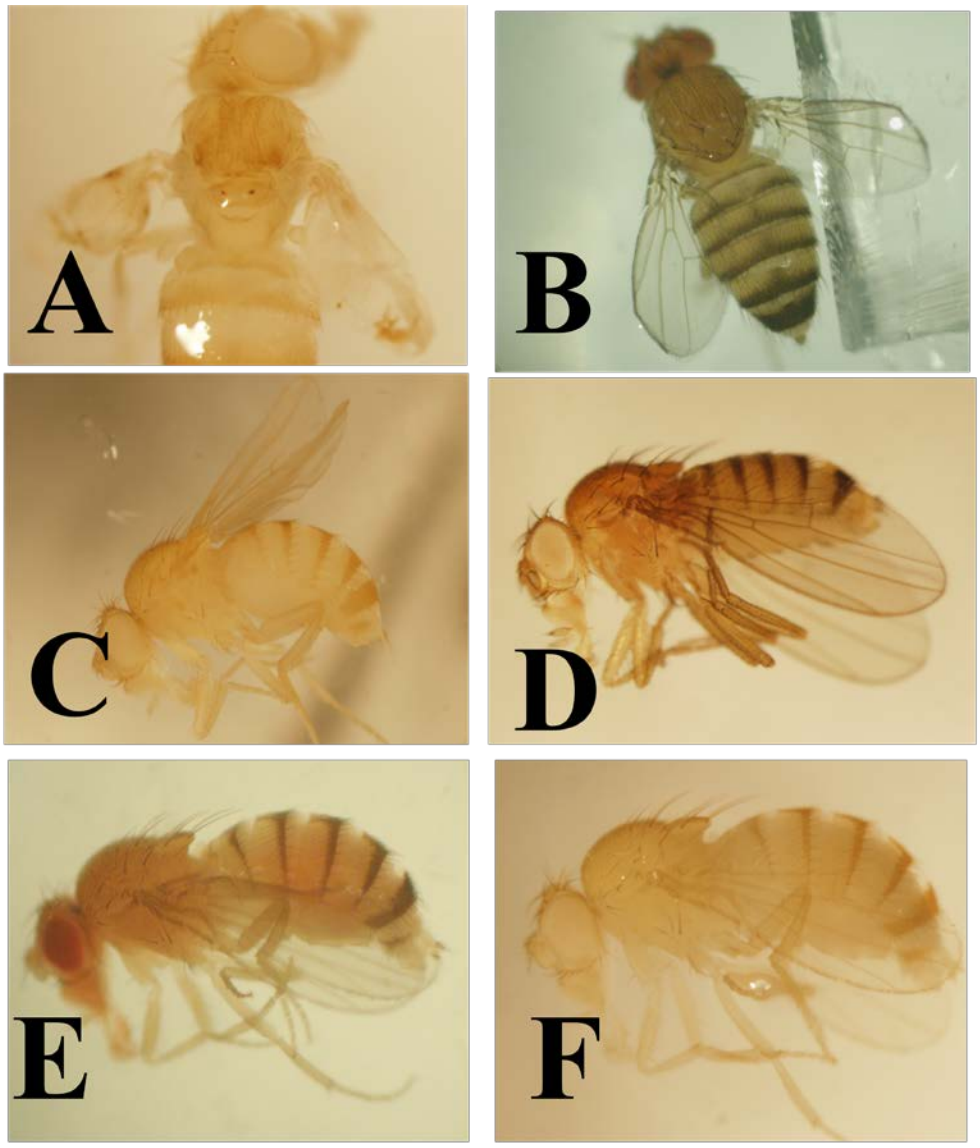

Figure 5: Identification of *fred* interacting genes

Animal (A) has the genetics of *ap-GAL4>>UAS-fits; UAS-cul-2-RNAi.* Notice the smaller wings and loss of macrochaetae compared to the control (B) *ap-GAL4 >>UAS-fits.* Animal (C) has the genetics *c765-GAL4>>UR3-1; UAS-cul-2-RNAi* and control (D) animal has *c765-GAL4>>UR3-1* genetics. Experimental animal (C) has smaller wings than control (D). The animal in *(E)* has the genetics of *c765-GAL4>>UR3-1; UAS-tum-RNAi* and control (F) has *c765-GAL4 >>UR3-1-fits.* The control in (F) has larger wings than the experimental animal (E).

Figure 6: *cul-2* knockdown associated phenotypes in adult animal

cul-2 knockdown leads to loss of microchaeta and macrochaeta in (A) *pnr-GAL4>>UAS-cul-2-RNAi*. Control animal (B) is without the *UAS* construct. Notice smaller wings with other drivers in (C,E, G). Animal (C) has driver *en-GAL4>>UAS-cul-2-RNAi*. Control (D) animal no *UAS*. Animal (E) has driver *ap-GAL4>>UAS-cul-2-RNAi*. Animal (F) is a control with no *UAS* construct. Animal in (G) has *c765-GAL4>>UAS-cul-2-RNAi*. Animal (H) is a control with no *UAS* construct.

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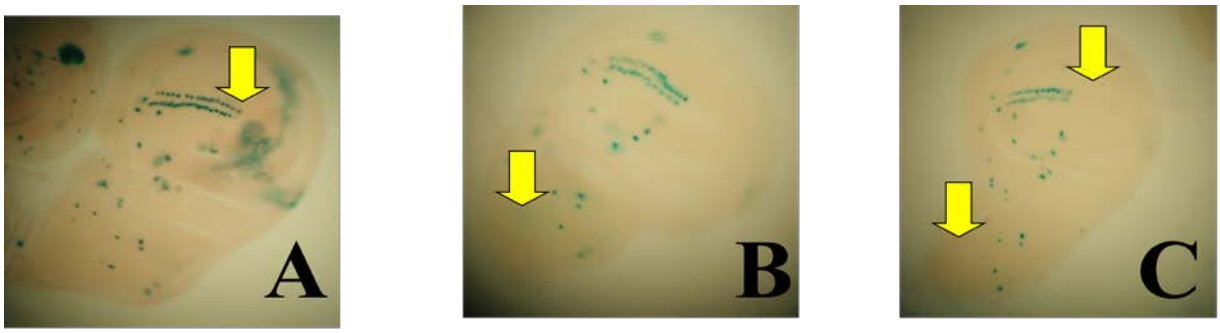


Figure 7: *cul-2* knockdown associated phenotypes in wing disc

neur-lacZ staining of wing discs with genotype (A) *en-GAL4>>UAS-Cul-2RNAi*. The wing disc (B) has genetics of *pnr-GAL4>>UAS-Cul-2RNAi*. The control in (C) has *neur-lacZ*. Notice in (A) and (B) ectopic SOPs compared to control (C).

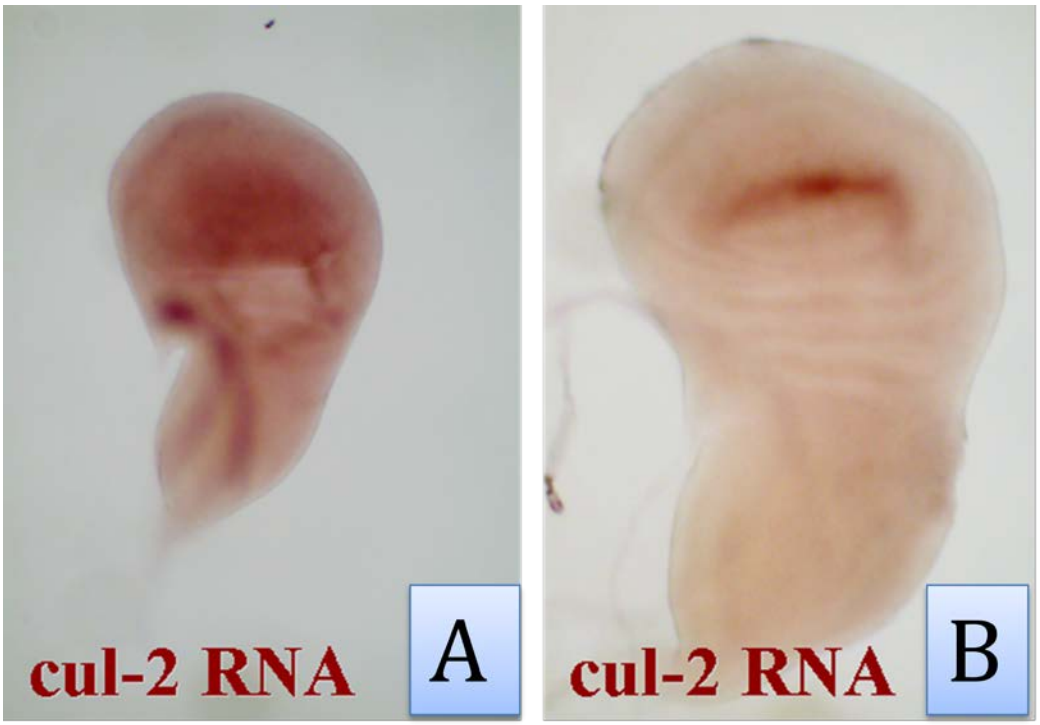


Figure 8: ***cul-2 in-situ***

In 1st day third instar larva (A) we find general uniform expression in the wing disc compared to second day third instar larva (B) where the wing disc shows lower general expression with higher expression in the wing margin.

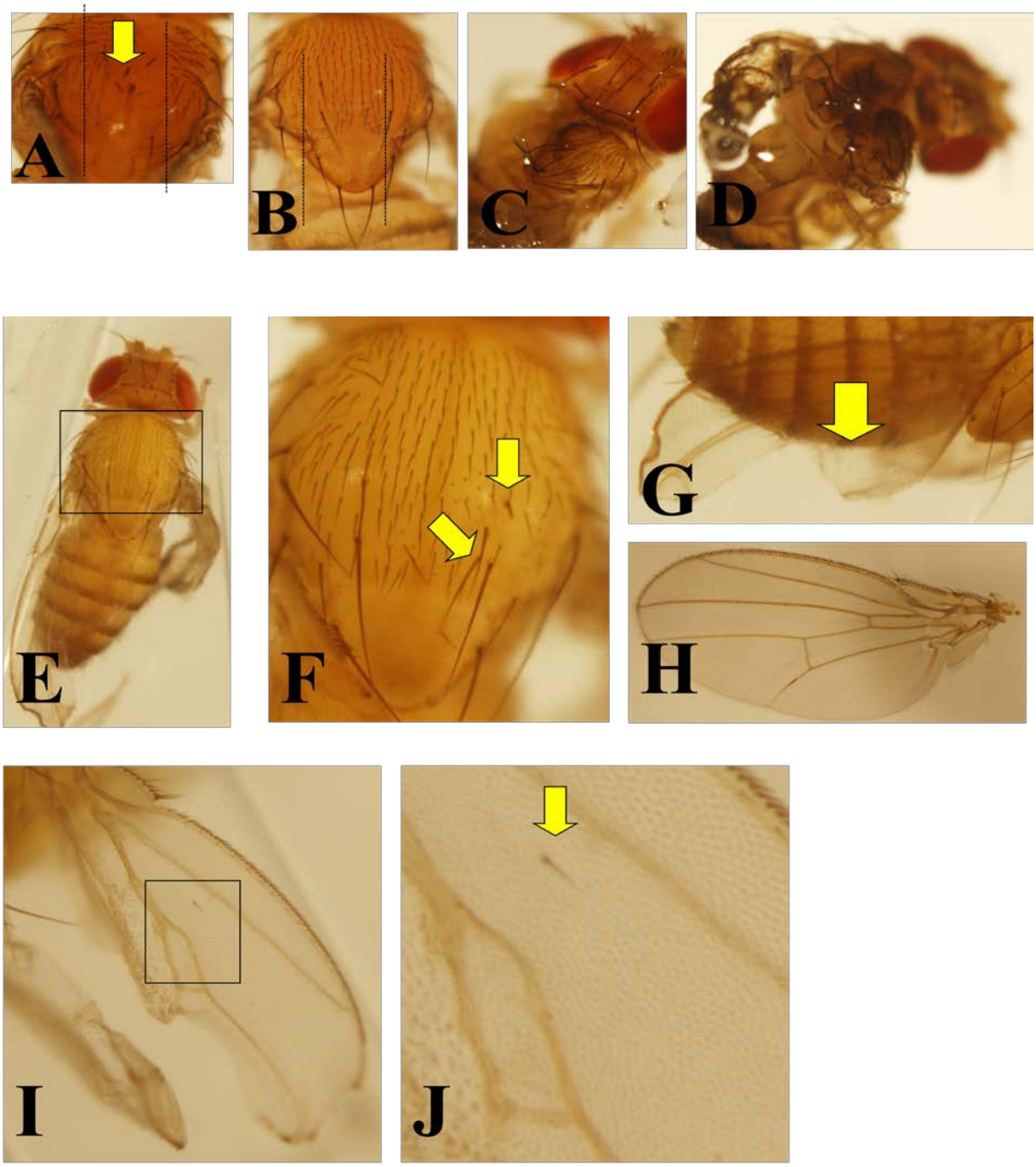


Figure 9: *tum* adult phenotypes

Suppression of *tum* in dorsal-central region with genotype *pnr-GAL4>>UAS-tum-RNAi* (A) displays macrochaeta and loss of epidermis. Wild type fly in (B and H). Flies with genotype *en-GAL4>>UAS-tum-RNAi* are depicted in (C-J excluding H). Notice the much stronger phenotypes in notum (C and D) compared to (F) and (I) animals.

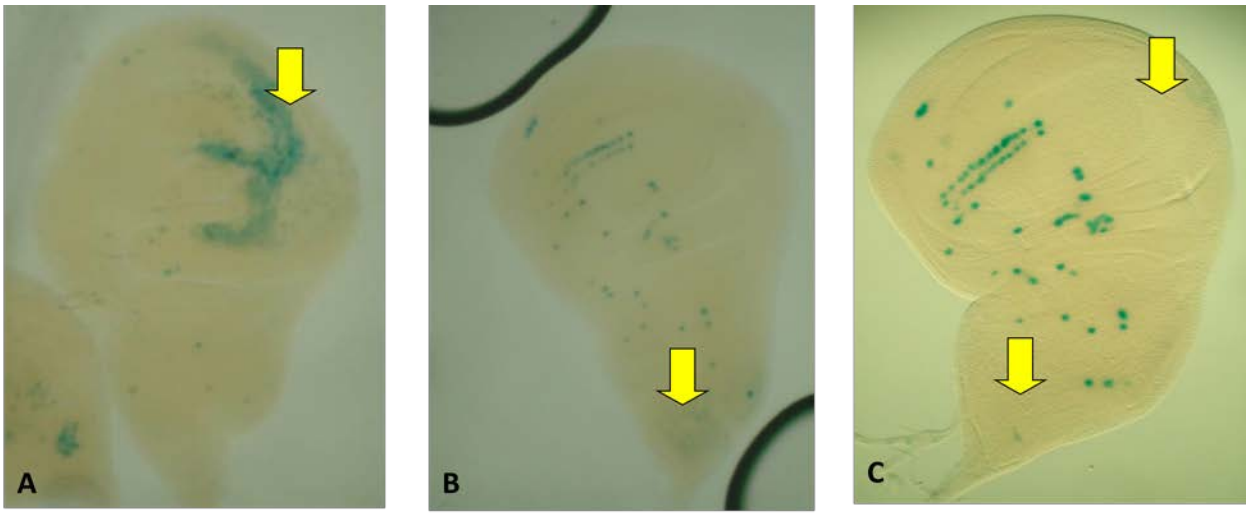
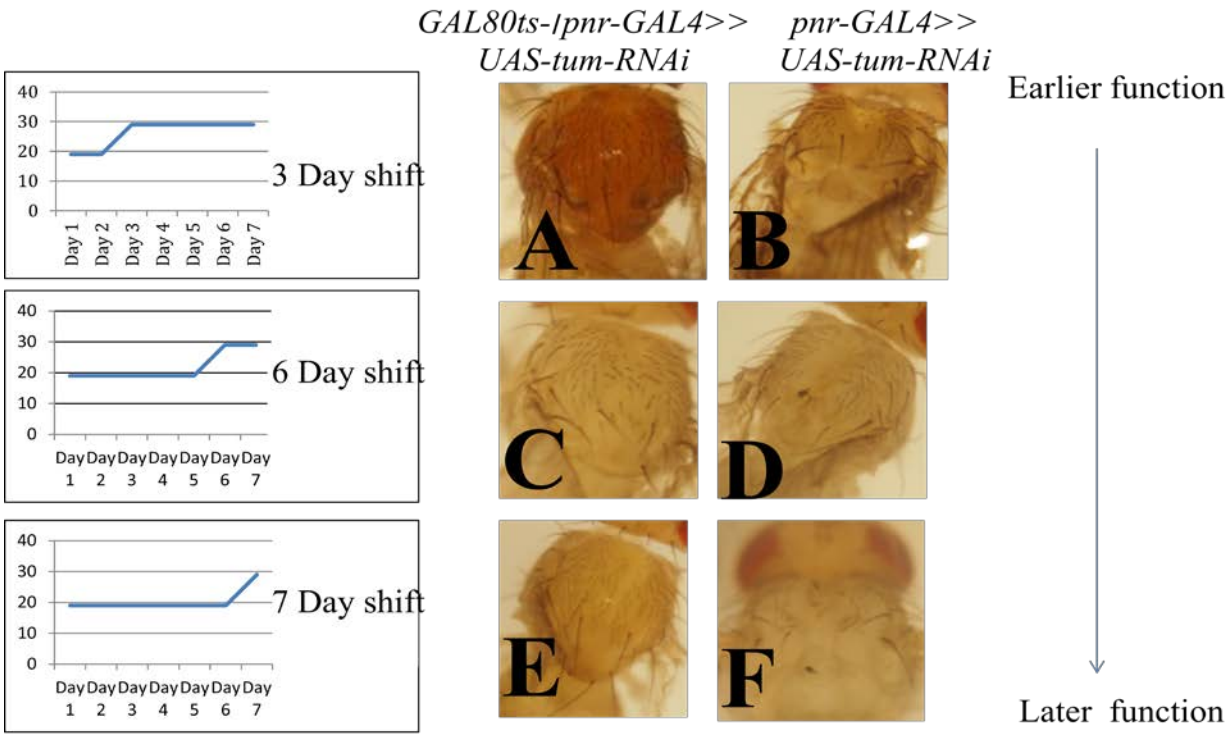


Figure 10: *tum* suppression in 3rd instar wing disc results in ectopic SOP formation

Ectopic SOPs evidence is shown in (A) wing disc with *en-GAL4>>UAS- tum-RNAi* genetics, and (B) *pnr-GAL4 >>UAS-tum-RNAi* genetics. Control wing disc (C) is shown for comparison. *neur-lacZ* staining was used in all cases above.



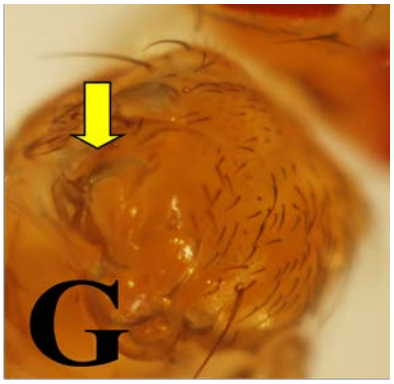


Figure 11: *tum* GAL80ts temperature shift study driver points to possible late function of t*um*

Animal in (A,C,E) have the genetics of *GAL80ts-/pnr-GAL4>>-UAS-tum-RNAi.* Animal in (B,D, F) have the genetics of *pnr-GAL4>>UAS-tum-RNAi* . The day shifted matches horizontally with the graphs and descend with possible later functions of *tum*. *GAL80ts* animals show polarity differences, and less bristles than the no *GAL80ts* animals. Fly (G) depicts one later function of *tum,* bulges at the notum.

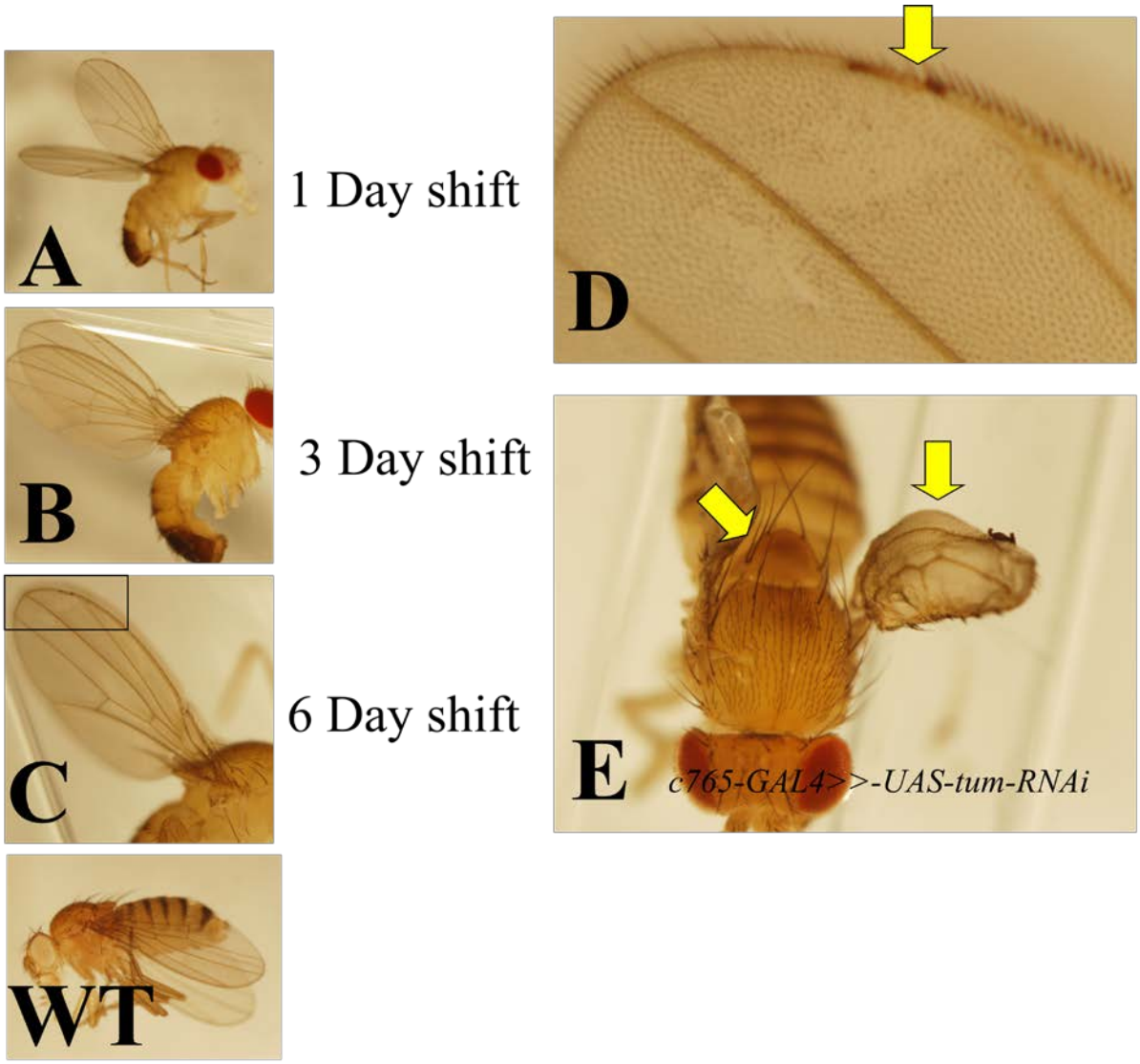


Figure 12: tum GAL80 temperature shift study driver c765-GAL4

Animal with *GAL80ts* (A-C) show 19°C to 29°C shift and partial upright wings. Wild type (WT) is the normal wing placement. Image (D) is a close up of (C). Later function of *tum* in wing marginal (D) is shown. Notice smaller wings, double bristle and socket in (E) of animal without GAL80ts .

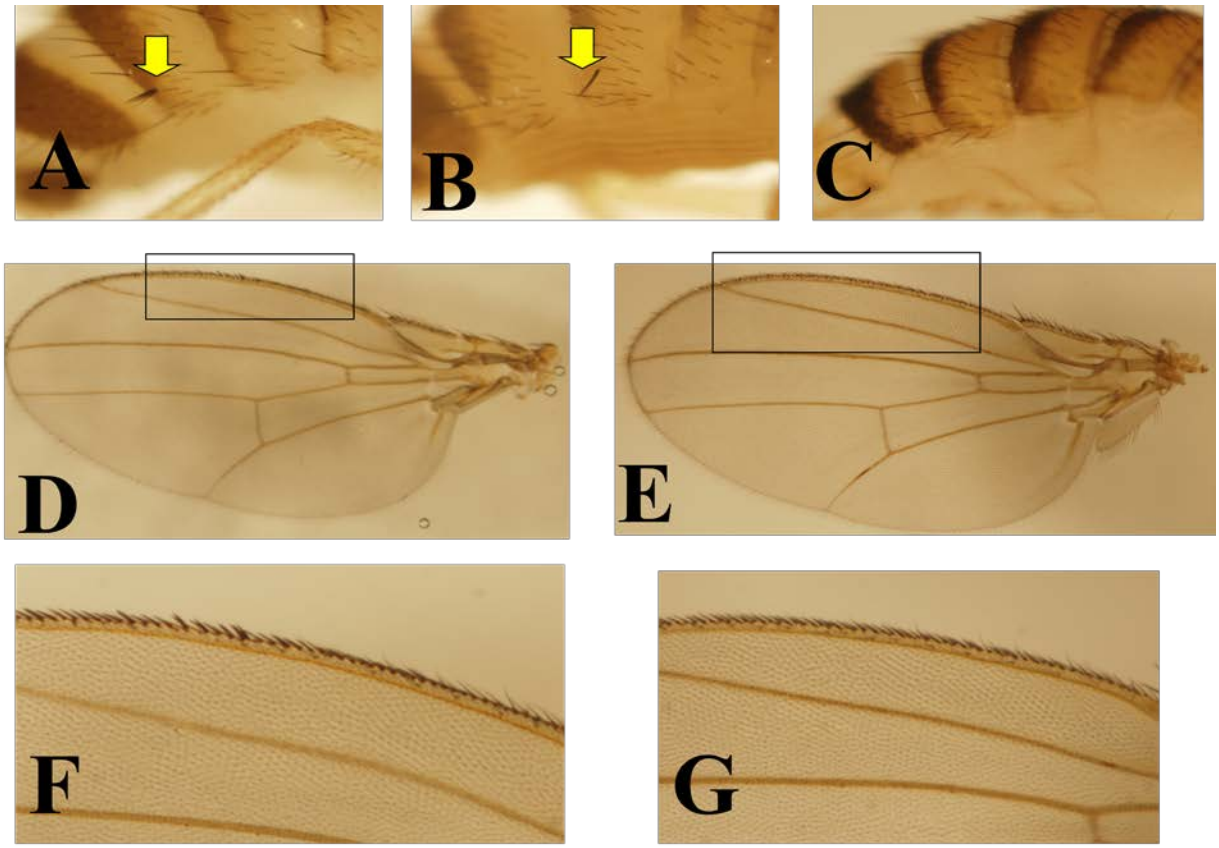


Figure 13: *tum* mosaic study

tum clone: *hsFLP; FRT42tum*^{347}*/FRT42-GFP*. Animal (A) shows cluster of bristles from a single socket. Animal (B) also shows a small clone. Animal (C, E, G) are wild type for comparison. Animal (D) is experimental wing. Image (F) is a close up of (D). Notice wing marginal phenotype in (F).

Discussion

cul-2 is required for sensory organ precursors for suppression of sensory organ formation

cul-2 encodes the scaffolding protein involved in ubiquitination. A total loss of *cul-2* results in non-development. Therefore *cul-2* must be knocked-down in certain parts of the fly at a time to be studied and then collectively compared. The purpose of this analysis was to determine and identify genes that might be involved in the *fred* pathway. To this end a set of fifteen lines were analyzed at the adult and third instar larva level. A total of two genes *tum* and *cul-2* were identified, both of which show qualitative similar phenotypes as *fred*. *fits* is a partial *fred* construct that contains only *fred* intercellular domain and transmembrane region. Expression of *fits* alone with *ap-GAL4* results in smaller wing phenotype. I used the sensitive *fred* background to see whether the RNAi mediated repression of candidate genes would modify this phenotype.

In following crosses with *pnr-GAL4, en-GAL4, c765-GAL4, ap-GAL4* drivers of different wing and/or notum expression, we have determined the phenotypes associated with *cul-2* knockdown. Specifically, I observed smaller wings, loss of macrochaeta, and a reduction of epidermis. These observations by themselves do not necessarily show that a *cul-2* knockdown is associated with SOP formation. To test this the SOP marker, *neur-lacZ,* which is specifically expressed in SOP cells, was used. *en-GAL4>>UAS-cul-2-RNAi* and *pnr-GAL4>>cul-2-RNAi* (figure 7) show ectopic A101 expression. These results mean that *cul-2* is required to suppress SOP formation in *Drosophila* wing disc cells. Additional genetic interaction studies involving Notch and *cul-2* draw conclusions that Notch signaling is not activated upon knock-down of *cul-2.* Experimental data for the ligand of Notch, Delta further supports this by being up regulated in a *UAS-sc* but not *UAS-cul-2.* (Zhang, Kusar, Vaessin; in revision).

tum is required for sensory organ precursors for suppression of sensory organ formation

Knock-down of *tum* with the *pnr-GAL4* and *en-GAL4* drivers (Figure 9) show animals with a loss of bristles on the notum and missing wing regions, respectively. The wing phenotype for the *tum-RNAi* line the *en-GAL4* driver is different from that observed for *cul-2-RNAi*. In the *cul-2-RNAi* fly, the wing is smaller and distorted. In the *tum* animal, part of the posterior compartment is missing with a more jagged appearance. However at the imaginal wing disc stage both *cul-2-RNAi* and *tum-RNAi* show ectopic A101 expression and SOP formation when expressed with *en-GAL4*. *tum* is required for limited SOP development. In other words ectopic SOP arise when *tum* expression levels are decreased or absent.

tum has a later function in development.

The temperature study of *tum* showed interesting results. There are two limitations in the genetic tools being used to better understand the results. First, *pnr-GAL4* and *c765-GAL4* both have different degrees of strength in driving expression of target constructs, with *pnr-GAL4* apparently being stronger. Second, these animals have balancers, which when you add anything that is not a wild type makes the animal sick to some degree. Furthermore, by stressing the animals at a higher temperature to inactivate *GAL80ts* can further cause phenotypes that may be contributed to what I will call the balancer effect. These limitations should be considered in the interpretations of the results.

In comparison to wild type animals the temperature suppression using *GAL-80ts* showed a later function of *tum* (Figure 11, 12). One sees two phenomena later in development from *tum* suppression with the *c765-GAL4* driver. First the “regular suppression” with GAL80ts at later shifts show bigger wings compared to “unmodified expression”, no GAL80ts. This means *tum* has an early function. However, “regular suppression” shows mild phenotypes of thicker bristles

in the wing marginal. This points to a later function of *tum* in addition to an early function. The second observation to note is that the animals did show a phenotype that may be related to a muscle attachment pathway because "regular suppression" flies showed defects in the positioning of the wings in comparison to the "wild type". Many of the wings pointed upwards in the "regular suppression". Conversely, "wild type" are laying flat. This could indicate a muscle innovation. This will have to be explored later.

GAL80ts-/pnr-GAL4>>UAS-tum-RNAi, on the other hand, showed different phenotypes. One interesting new phenotype observed was that underneath the notum were what appeared to be bulges in the epidermis of unknown origin in the "regular suppression" animals (Figure 11). "Regular suppression" animals had loss of macro and microchaetae on the median notum compared to "wild type". "Unmodified expression" displayed loss of epidermis, and bristle duplication.

tum has a function in bristle formation

Interesting bristle phenotypes appear throughout the study of *cul-2* and *tum*. The adult bristle is composed of four cells: the neuron, socket, shaft and sheath (Jarman, 2002). All four cells derive two or three cell divisions from an SOP cell in the imaginal disc. With this in mind, a possible explanation of the phenotypes could be the transformation of one cell into another causing some of the phenotypes observed, such as three bristles (Figure 13), and double socket and bristle (Figure 12). These types of phenotypes have been observed in several mutants that function during later stages of sensory organ formation (Song and Lu 2012).

ModENCODE Temporal Expression Data from fly base further suggest that *tum* has a later function. It appears that *tum* has a very high expression from 0-8 hours in development but lower levels during first and second instar larva and moderate levels at third instar, and high

levels in adult females (Graveley et al., 2011). Also to note is the expression of *tum* in organ and tissue is of moderate expression in CNS during larvae and ovary in adult (Chintapalli et al., 2007).

In the mosaic analysis of *tum*, the FLP/FRT system previously discussed in this thesis was utilized for mosaic/colonel analysis of *tum*. Colonel analysis was used to gain independent insight in the specificity of *tum* gene function. The initial analysis showed animals with various sensory organ phenotypes such as 3 shaft cells protruding at a single point. Further studies would need to be done to trace the cells that make up a bristle to determine what cells are transforming into this multiple shaft bristle. The results observed so far are in line with role of tum in SOP and sensory organ formation.

***tum* in *fred, tum* in *cul-2,* and *tum* in *sc* interaction**

Both *tum* and *fred* down regulated by the *en-GAL4* driver in the same animal result in animals that have the posterior part of the wing missing, but in a smooth appearance compared to the single mutation of *tum*. This different phenotype was so far not universal among all animals, to clearly state whether there are epistatic interactions between *tum* and *fred,*or if they act in more of an additive observation. Further testing will need to be done to determine this.

The *tum* and *cul-2* double mutation down regulated by the *en-GAL4* driver showed much variability. One explanation would be that each mutation by itself would have a level of variability in the progeny. Often it is the animal with the weakest down regulation that survives. However, when two mutations that have variability are combined the progeny may have the potential to have even greater variability. This could help explain why some animals had small wings, while other animals had even small wings. Others still had what looked like a gain of wing veins. From this range of observation the same conclusion of the *tum* and *fred* mutation

resulted. There is inconclusive evidence at this point to argue how *tum* and *cul-2* interact in relations to one another in development.

The last double mutation genotype tested with *en-GAL4* driver was *tum-RNAi* and *sc-RNAi*. Achaete-scute complex (ASC) is a proneural gene in wing discs that provides SOP potential. The complex is made of four basic Helix-Loop-Helix (bHLH) transcription factors one which is *scute* (Vaessin et al., 1994). Previous studies of *cul-2* and *sc* showed *cul-2*-associated ectopic SOP requires the activity of the proneural *acheatescute complex* gene *scute* (Zhong, Kusasr, Vaessin, in revision). If the results of *tum* and *sc* are anything like *cul-2* and *sc* one would suspect loss of ectopic SOP. However the results are mixed. Some wing disc show ectopic SOP, while others do not. Further testing will need to be done to reach a more conclusive result.

Conclusion

Cul-2 and *tum* are required to prevent inappropriate sensory organ formation in Drosophila. Both genes prevent epidermal cells from becoming neurogenic fates in the wing disc. The *cul-2* and *tum* losses of function phenotypes closely resemble the *fred* loss of function phenotype. *Tum* has a later function in development. Future work will have to determine the potential roles of *cul-2* and *tum* in relation to *fred* function.

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